DESCRIPTION

AN EFFICIENT NON-VIRAL GENE/DRUG DELIVERY SYSTEM

BACKGROUND OF THE INVENTION

The present application claims priority to co-pending U. S. Provisional Patent Application Serial No. 60/436,174 filed on December 23, 2002. The entire text of the above-referenced disclosure is specifically incorporated herein by reference without disclaimer.

1. Field of the Invention

The present invention relates generally to the fields of cancer biology and drug delivery systems. More particularly, it concerns an efficient method for delivering a molecule or agent to a cell.

2. Description of Related Art

The development of new forms of therapeutics which use macromolecules (proteins, nucleic acids, *etc.*) has created a need to develop effective means of delivering such molecules to their appropriate cellular targets. Therapeutics based on either the use of specific polypeptide growth factors or specific genes to replace or supplement absent or defective genes are examples of therapeutics which may require such new delivery systems. Clinical application of such therapies depends not only on the efficacy of new delivery systems, but also on their safety and on the ease with which the technologies underlying these systems can be adapted for large scale pharmaceutical production, storage, and distribution of the therapeutic formulations.

Gene therapy has become an increasingly important mode of treating a variety of diseases and disorders. The potential for providing effective treatments, and even cures, has stimulated an intense effort to apply this technology to diseases for which there have been no effective treatments. Recent progress in this area has indicated that gene therapy may have a significant impact, not only on the treatment of single gene disorders, but also on other more complex diseases such as cancer. However, a significant obstacle in the attainment of efficient gene therapy has been the difficulty of designing new and effective means of delivering therapeutic nucleic acids to cell targets.

Viral vectors are another delivery system known in the art. These systems are relatively efficient gene delivery systems, for *in vitro* or local administration *in vivo*, but suffer from a

variety of limitations. These limitations include the potential for reversion to the wild-type as well as immune response concerns, and inefficiency in systemic gene delivery *in vivo*, *e.g.*, it is inefficient after intravenous injection. As a result, non-viral gene delivery systems are receiving increasing attention (Worgall *et al.*, 1997; Peeters *et al.*, 1996; Yei *et al.*, 1994; Hope *et al.*, 1998). Plasmid DNA-cationic liposome complexes are currently the most commonly employed non-viral gene delivery vehicles (Felgner, 1997; Chonn *et al.*, 1995). However, complexes are large, poorly defined systems that are not suited for systemic applications and can elicit considerable toxic side effects (Harrison *et al.*, 1995; Huang *et al.*, 1997; Templeton *et al.*, 1997; Hofland *et al.*, 1997.

A variety of formulations, such as cationic liposome formulations, have been utilized as delivery systems. Such cationic liposome formulations known in the art, include the commercially available cationic liposome reagent DOTMA/DOPE (N-1, -(2,3-dioleoyloxy) propyl-N,N,N-trimethyl ammonium chloride/dioleoyl phosphatidylethanolamine) (Felgner *et al.*, 1987). Additionally, a cationic liposome formulation designated DC-Chol/DOPE (3-β-N-(N',N'-dimethylaminoethane)-carbamoyl cholesterol/(dioleoyl phosphatidylethanolamine) has been shown in *in vitro* studies (Gao and Huang, 1991) to be relatively nontoxic and more efficient than DOTMA/DOPE.

However, the use of DC-Chol/DOPE and other currently existing cationic liposomes as vehicles for delivering nucleic acids to cellular targets is inconvenient for large scale therapeutic applications for a number of reasons. Primarily, the ratios of liposome to nucleic acid utilized to form nucleic acid/liposome complex in the prior art admixture method results in the formation of complexes which are large in diameter and relatively unstable. Thus, none of the presently utilized cationic liposome formulations, including DC-Chol/DOPE, are designed as stable and ready-to-use pharmaceutical formulations of nucleic acid/liposome complex. This limitation of the admixture method requires that the user prepare the complex prior to each use.

Thus, an ideal system for the delivery of exogenous genes into cells and tissues should be highly efficient in nucleic acid delivery, safe to use, easy to produce in large quantity and have sufficient stability to be practicable as a pharmaceutical agent.

SUMMARY OF THE INVENTION

The present invention is directed to a therapeutic delivery system for delivering a molecule to a cell and overcomes the deficiencies in the art of current delivery systems in providing a molecule to a cell. The present invention provides a non-viral composition as an

alternative to viral gene therapies. The present invention addresses the need for an effective, safe and clinically useful gene delivery system.

Therefore, the present invention provides a method for preparing a delivery composition comprising admixing a first lipid comprising 1,2-diacyl-sn-glycero-3-ethylphosphocholine, a second lipid, and two or more cationic polymers, to produce a composition for delivering a selected molecule to a cell. The second lipid of the present invention may be a polymer-linked lipid such as 1,2-diacyl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] or 1,2-diacyl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]. In all embodiments of the present invention, the first lipid is distinct from the second lipid.

In some embodiments, cationic polymers of the delivery composition are polyethyleneimine and protamine. In a particular embodiment, the ratio of polyethyleneimine to protamine is between about 0.01:1 and about 0.2:1. In a further particular embodiment, the ratio of polyethyleneimine to protamine is about 0.02:1.

In another embodiment, cationic polymers of the delivery composition are polylysine and protamine. In a particular embodiment, the ratio of polylysine to protamine is between about 0.1:1 and about 1:1. In a further particular embodiment, the ratio of polylysine to protamine is about 0.3 to 1.

In still another embodiment, cationic polymers of the delivery composition are polyethyleneimine, protamine and polylysine. In particular embodiments, the ratio of polyethyleneimine to protamine to polylysine is between about 0.002:0.05:1 and about 0.02:0.5:1. In a further particular embodiment, the ratio of polyethyleneimine to protamine to polylysine is about 0.01:0.3:1.

In other aspects of the invention, the ratio of the cationic polymer and the selected molecule is between about 2:0.1 and about 10:5; the ratio of the second lipid and the selected molecule is between about 0.2:0.1 and about 5:5; and the ratio of 1,2-diacyl-sn-glycero-3-ethylphosphocholine and the selected molecule is between about 0.2:0.1 and about 10:5.

In particular embodiments of the invention, the selected molecule is a DNA, a oligonucleotide, a RNA, a protein or a drug such as a chemotherapeutic agent. In other particular aspects of the invention the selected molecule may be an expression construct which expresses a biologically functional protein or peptide such as a tumor suppressor, a tumor suppressor activator, a pro-apoptotic factor or gene, an oncogenic blocker, or an anti-angiogenesis factor but is not limited to such. In particular embodiments, the tumor suppressor may be p53.

In still another aspect of the invention, the cell is a cancer cell or a macrophage cell. In further embodiments, the cancer cell may be a metastatic lung cancer cell, a head and neck cancer cell, a thyroid cancer cell, a liver cancer cell, a breast cancer cell, a prostate cancer cell, a ovarian cancer cell, a colon cancer cell, a rectum cancer cell, a pancreas cancer cell, a spleen cancer cell, a stomach cancer cell, a duodenum cancer cell, a kidney cancer cell, a uterus cancer cell, a cervical cancer cell, a testicle cancer cell, a brain cancer cell, a bone cancer cell, a lymphoid cancer cell, a skin cancer cell, or a vesicular cancer cell. In a further embodiment, the metastatic lung cancer cell may be a small cell lung carcinoma cell.

The present invention also provides a method for delivering a selected molecule to a cell comprising (a) providing a composition comprising: (i) a first lipid comprising 1,2-diacyl-sn-glycero-3-ethylphosphocholine; (ii) a second lipid; (iii) two or more cationic polymers; (iv) the selected molecule, and (b) contacting the cell with the composition. In all embodiments of the invention, the first lipid is distinct from the second lipid.

In some embodiments of the invention, the cell may be in a subject such as a mammal. In further embodiments, the mammal is a human.

The present invention further provides a method for treating cancer comprising administering to a subject a composition comprising (a) a first lipid comprising 1,2-diacyl-sn-glycero-3-ethylphosphocholine; (b) a second lipid; (c) two or more cationic polymers; and (d) a therapeutically effective amount of an anticancer agent. In other embodiments of the invention, the cancer may be a primary or metastatic cancer such as a lung cancer, a head and neck cancer, a thyroid cancer, a liver cancer, a breast cancer, a prostate cancer, a ovarian cancer, a colon cancer, a rectum cancer, a pancreas cancer, a spleen cancer, a stomach cancer, a duodenum cancer, a kidney cancer, a uterus cancer, a cervical cancer, a testicle cancer, a brain cancer, a bone cancer, a lymphoid cancer, a skin cancer cell, or a vesicular cancer cell but is not limited to such. In a further embodiment, the metastatic lung cancer may be a small cell lung carcinoma.

In particular embodiments of the invention, the administering may be intravenously, intraperitoneally, intratracheally, or by inhalation but is not limited to such. In other embodiments the administering may be once or more than once.

In further embodiments, the anticancer agent may be a DNA, a oligonucleotide, a RNA, a protein or a drug such as a chemotherapeutic agent.

In still further embodiments the anticancer agent may be an expression construct which expresses a biologically functional protein or peptide such as a tumor suppressor gene, a tumor suppressor activator, a pro-apoptotic factor or gene, an oncogenic blocker or an anti -

angiogenesis factor but is not limited to such. In a particular embodiment, the tumor suppressor gene may be p53.

In still yet a further particular embodiment the present invention provides a composition comprising (a) a first lipid comprising 1,2-diacyl-sn-glycero-3-ethylphosphocholine; (b) a second lipid; and (c) two or more cationic polymers. In all embodiments of the present invention, the first lipid is distinct from the second lipid. In particular embodiments, the composition of the present invention further comprises an agent

The second lipid of the composition may be a polymer-linked lipid such as 1,2-diacyl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] or 1,2-diacyl-sn-glycerol-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000].

In some embodiments, cationic polymers of the composition are polyethyleneimine and protamine. In particular embodiments, the ratio of polyethyleneimine to protamine is between about 0.01:1 and about 0.2:1. In a further particular embodiment, the ratio of polyethyleneimine to protamine is about 0.02:1.

In yet another embodiment, cationic polymers of the composition are polylysine and protamine. In still another particular embodiment, the ratio of polylysine to protamine is between about 0.1:1 and about 1:1. In a further particular embodiment, the ratio of polylysine to protamine is about 0.3 to 1.

In still another embodiment, cationic polymers of the composition are polyethyleneimine, protamine and polylysine. In particular embodiments, the ratio of polyethyleneimine to protamine to polylysine is between about 0.002:0.05:1 and about 0.02:0.5:1. In a further particular embodiment the ratio of polyethyleneimine to protamine to polylysine is about 0.01:0.3:1.

In other aspects of the invention, the ratio of the cationic polymer and the agent is between about 2:0.1 and about 10:5; the ratio of the second lipid and the agent is between about 0.2:0.1 and about 5:5; and the ratio of 1,2-diacyl-sn-glycero-3-ethylphosphocholine and the selected molecule is between about 0.2:0.1 and about 10:5.

In particular embodiments of the invention, the agent may be a DNA, a oligonucleotide, a RNA, a protein or a drug such as a chemotherapeutic agent. In other particular aspects of the invention the agent may be an expression construct which expresses a biologically functional protein or peptide such as a tumor suppressor, a tumor suppressor activator, a pro-apoptotic factor or gene, an oncogenic blocker, or an anti-angiogenesis factor but is not limited to such. In particular embodiments, the tumor suppressor may be p53.

It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Transfection efficiency of ENGD-GFP as compared to lipofectamine and lipofectin.

FIG. 2. Sustained release function of ENGD compared with non-polymer liposomes.

FIG. 3. The size of ENGD-DNA complex particles in formulations.

FIGS. 4A-4B. ENGD efficiently delivers the reporter gene into the solid tumor and other organs in mice through intravenous injection. **FIG. 4A.** The tissue distribution of ENGD encapsulated luciferase gene in nude mice subcutaneously implanted with head and neck cancer (KB) cell line. **FIG. 4B.** The tissue distribution of ENGD encapsulated luciferase gene in nude mice subcutaneously implanted with human non-small cell lung carcinoma (H358) cell line.

FIGS. 5A-5C. Systemically injected ENGD-p53 significantly inhibits growth of human solid tumors implanted in nude mice. **FIG. 5A** - i.v. injected ENGD1-p53 and ENGD5-p53 significantly inhibited growth in human head and neck cancer (kb) implanted subcutaneously in nude mice; lipofectamine-p53 was not effective. **FIG. 5B** - i.v. injected ENGD1-p53 and ENGD5-p53 significantly inhibited growth in human lung cancer (h358) implanted subcutaneously in nude mice; lipofectamine-p53 was not effective. **FIG. 5C** - i.v. injected

ENGD1-p53 and ENGD5-p53 significantly inhibited growth in orthotopic breast cancer (kb) implanted subcutaneously in nude mice.

FIG. 6. Delivery of a CMV-promoter driven luciferase gene encapsulated in ENGD (ENGD-*luc*) to metastatic SCLC tumors using an *in vivo* mouse model.

FIG.7. Delivery of a small chemical molecule encapsulated in ENGD to metastatic SCLC tumors using an *in vivo* mouse model.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

An ideal system for the delivery of macromolecules and micromolecules into cells and tissues should be highly efficient, safe to use, easy to produce in large quantity and have sufficient stability to be practicable as a pharmaceutical agent. Formulations of the present invention provide such as system.

A. The Present Invention

The present invention provides a non-viral system for delivery of a molecule or agent to a cell. This system provides several advantages over the prior art. One such advantage is that vector DNA can be widely and routinely prepared on a large scale, eliminating the risk of using viral vectors such as retroviruses. Another advantage of this non-viral system is that liposome-mediated gene delivery, unlike retroviral-mediated gene delivery, can deliver either RNA or DNA. Thus, DNA, RNA, or an oligonucleotide can be introduced directly into a cell. Moreover, cationic liposomes, which are employed in the present invention, are non-toxic, non-immunogenic and can therefore be used repeatedly *in vivo* as evidenced by the successful *in vivo* delivery of genes to catheterized blood vessels (Nabel *et al.*, 1990), and lung epithelial cells (Brigham *et al.*, 1989; Stribling *et al.*, 1992). Other systemic uses of cationic liposomes have also been reported (Zhu *et al.*, 1993; Philip *et al.*, 1993; Nabel *et al.*, 1994).

The delivery composition of the present invention utilizes PEG-lated lipids in the formulation, which stabilizes liposomes for delivery of such molecules as chemotherapeutic agents. The present invention provides a delivery system which encapsulates a molecule such as, for example, DNA or a drug in the stabilized liposomes thereby protecting the molecule from degradation. The present invention further provides a delivery system which first condenses a molecule such as DNA using cationic polymers to make a small polymer-DNA complex, and then encapsulates the polymer-DNA particles in the stabilized liposomes.

The delivery composition of the present invention differs from those in the art in that it utilizes multiple cationic polymers to generate a more efficient delivery system whereas, single cationic polymers are known to be used in the art. Thus, the ratios of the combination of the different polymers are an important aspect of the present invention.

The present invention also provides an efficient delivery system for targeting a gene or drug by adding targeting ligands on the surface of the liposomes. These ligands can be tumor-targeted peptides, specific antibodies, or any other targeting compound. The present invention further provides a system for efficiently delivering a reporter gene into an implanted tumor, or to various organs of a subject via intravenous injection.

Overall, the present invention comprises a stabilized lipid, a cationic lipid, and multiple cationic polymers to provide an efficient gene delivery system for systemic gene delivery *in vivo*, especially for intravenous gene delivery in treating various cancers.

B. Polycations and Polymers

Polycations have been utilized in the art to facilitate delivery of nucleic acids to the interior of cells both *in vitro* and *in vivo*. Such polycations include polylysine, protamines, histones, spermine, spermidine, polyornithine, polyargnine, and putrescine. Polycations may also include synthetic polycations based on acrylamide and 2-acrylamido-2-methylpropanetrimethylamine, poly(N-ethyl-4-vinylpyridine) or similar quarternized polypyridine, diethylaminoethyl polymers and dextran conjugates, polymyxin B sulfate, lipopolyamines, poly(allylamines) such as the strong polycation poly(dimethyidiallylammonium chloride), polybrene, spermine, spermidine and polypeptides such as protamine, the histone polypeptides, polylysine, polyarginine and polyornithine. When polyamino acids such as polylysine and polyarginine are used, preferred sizes are from about 10 to about 200 residues.

Particularly preferred polycations of the present invention are polylysine, protamine, and polyethyleneimine.

Polymers are also utilized in the art in facilitating delivery of a molecule to a cell. Such polymers may be natural or synthetic polymers and amy be employed in the composition of the present invention. Synthetic polymers are usually preferred due to the better characterization of degradation and release profiles. A polymer is selected based on the period over which release is desired, generally in the range of at least three months to twelve months, although longer periods may be desirable. In some cases linear release may be most useful, although in others a pulse release or "bulk release" may provided more effective results. The polymer may be in the form

of a hydrogel (typically in absorbing up to about 90% by weight of water), and can optionally be crosslinked with multivalent ions or polymers.

Polymers may include soluble linear or branched polymers. Soluble polymers are polymers that are soluble in a water miscible solvent or aqueous solution. Polymers may be solubilized by first being dissolved in an organic solvent and then combined with an aqueous solvent.

Polymers comprise categories such as (1) carbohydrate-based polymers, such as methycellulose, carboxymethyl cellulose-based polymers, dextran, polydextrose, chitins, chitosan, and starch (including hetastarch), and derivatives thereof; (2) polyaliphatic alcohol such as polyethylene oxide and derivatives thereof, including: polyethylene glycol (PEG), PEG-acrylates, polyethyleneimine, polyvinyl acetate, and derivatives thereof; (3) poly(vinyl) polymers such as poly(vinyl) alcohol, poly(vinyl)pyrrolidone, poly(vinyl)phosphate, poly(vinyl)phosphonic acid, and derivatives thereof; (4) polyacrylic acids and derivatives thereof; (5) polyorganic acids, such as polymaleic acid, and derivatives thereof; (6) polyamino acids, such as polylysine, and polyimino acids, such as polyimino tyrosine, and derivatives thereof; (7) co-polymers and block co-polymers, such as poloxamer 407 or Pluronic L-101™ polymer, and derivatives thereof; (8) tert-polymers and derivatives thereof; (9) polyethers, such as poly(tetramethylene ether glycol), and derivatives thereof; (10) naturally occurring polymers, such as zein, chitosan, pullulan, and derivatives thereof; (11) polyimids, such as poly n-tris(hydroxymethyl)methylmethacrylate, and derivatives thereof; (12) surfactants, such as polyoxyethylene sorbitan, and derivatives thereof; (13) polyesters such as poly(ethylene glycol)(n)monomethyl ether mono(succinimidyl succinate)ester, and derivatives thereof; (14) branched and cyclo-polymers, such as branched PEG and cyclodextrins, and derivatives thereof; and (15) polyaldehydes, such as poly(perfluoropropylene oxide-b-perfluoroformaldehyde), and derivatives thereof.

A polymer or polymer mixture may be prepared in accordance with the methods set forth in U.S. Patent 5,525,519 or PCT Patent WO 93/14110), both of which are incorporated herein by reference, in which the polymer is dissolved in water or an aqueous solution, such as a buffer, in a concentration between approximately 1 and 50 g/100 ml depending on the molecular weight of the polymer.

C. Lipid Compositions

In all embodiments, the present invention concerns a novel delivery composition partly comprising lipids. A lipid is a substance that is characteristically insoluble in water and

extractable with an organic solvent. Any lipid, as is known to one of ordinary skill in the art, is encompassed by the compositions and methods of the present invention.

A lipid may be naturally occurring or synthetic (*i.e.*, designed or produced by man). However, a lipid is usually a biological substance. Biological lipids are well known in the art, and include for example, neutral fats, phospholipids, phosphoglycerides, steroids, terpenes, lysolipids, glycosphingolipids, glucolipids, sulphatides, lipids with ether and ester-linked fatty acids and polymerizable lipids, and combinations thereof.

1. Lipid Types

A neutral fat as may be employed in the composition of the present invention, may comprise a glycerol and a fatty acid. A typical glycerol is a three carbon alcohol. A fatty acid generally is a molecule comprising a carbon chain with an acidic moiety (*e.g.*, carboxylic acid) at an end of the chain. The carbon chain of a fatty acid may be of any length, however, it is preferred that the length of the carbon chain be of from about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, to about 30 or more carbon atoms, and any range derivable therein. However, a preferred range is from about 14 to about 24 carbon atoms in the chain portion of the fatty acid, with about 16 to about 18 carbon atoms being particularly preferred in certain embodiments. In certain embodiments the fatty acid carbon chain may comprise an odd number of carbon atoms, however, an even number of carbon atoms in the chain may be preferred in certain embodiments. A fatty acid comprising only single bonds in its carbon chain is called saturated, while a fatty acid comprising at least one double bond in its chain is called unsaturated.

Specific fatty acids include, but are not limited to, linoleic acid, oleic acid, palmitic acid, linolenic acid, stearic acid, lauric acid, myristic acid, arachidic acid, palmitoleic acid, arachidonic acid ricinoleic acid, tuberculosteric acid, and lactobacillic acid. An acidic group of one or more fatty acids is covalently bonded to one or more hydroxyl groups of a glycerol. Thus, a monoglyceride comprises a glycerol and one fatty acid, a diglyceride comprises a glycerol and two fatty acids, and a triglyceride comprises a glycerol and three fatty acids.

A phospholipid as may be employed in the composition of the present invention, generally comprises either glycerol or an sphingosine moiety, an ionic phosphate group to produce an amphipathic compound, and one or more fatty acids. Types of phospholipids include, for example, phophoglycerides, wherein a phosphate group is linked to the first carbon

of glycerol of a diglyceride, and sphingophospholipids (*e.g.*, sphingomyelin), wherein a phosphate group is esterified to a sphingosine amino alcohol. Another example of a sphingophospholipid is a sulfatide, which comprises an ionic sulfate group that makes the molecule amphipathic. A phopholipid may, of course, comprise further chemical groups, such as for example, an alcohol attached to the phosphate group. Examples of such alcohol groups include serine, ethanolamine, choline, glycerol and inositol. Thus, specific phosphoglycerides include a phosphatidyl serine, a phosphatidyl ethanolamine, a phosphatidyl choline, a phosphatidyl glycerol or a phosphotidyl inositol. Other phospholipids include a phosphatidic acid or a diacetyl phosphate. A phosphatidylcholine comprises a dioleoylphosphatidylcholine (*a.k.a.* cardiolipin), an egg phosphatidylcholine, a dipalmitoyl phosphalidycholine, a monomyristoyl phosphatidylcholine, a monopalmitoyl phosphatidylcholine, a monostearoyl phosphatidylcholine, a monooleoyl phosphatidylcholine, a dibutroyl phosphatidylcholine, a divaleroyl phosphatidylcholine, a dicaproyl phosphatidylcholine, a diheptanoyl phosphatidylcholine, a dicapryloyl phosphatidylcholine or a distearoyl phosphatidylcholine.

A glycolipid as may be employed in the composition of the present invention, is related to a sphinogophospholipid, but comprises a carbohydrate group rather than a phosphate group attached to a primary hydroxyl group of the sphingosine. A type of glycolipid called a cerebroside comprises one sugar group (*e.g.*, a glucose or galactose) attached to the primary hydroxyl group. Another example of a glycolipid is a ganglioside (*e.g.*, a monosialoganglioside, a GM1), which comprises about 2, about 3, about 4, about 5, about 6, to about 7 or so sugar groups, that may be in a branched chain, attached to the primary hydroxyl group. Ceramide such as lactosylceramide also constitutes a glycolipid.

A steroid as may be employed in the composition of the present invention, is a four-membered ring system derivative of a phenanthrene. Steroids often possess regulatory functions in cells, tissues and organisms, and include, for example, hormones and related compounds in the progestagen (*e.g.*, progesterone), glucocoricoid (*e.g.*, cortisol), mineralocorticoid (*e.g.*, aldosterone), androgen (*e.g.*, testosterone) and estrogen (*e.g.*, estrone) families. Cholesterol is another example of a steroid, and generally serves structural rather than regulatory functions. Vitamin D is another example of a sterol, and is involved in calcium absorption from the intestine.

A terpene as may be employed in the composition of the present invention, is a lipid comprising one or more five carbon isoprene groups. Terpenes have various biological functions, and include, for example, vitamin A, coenyzme Q and carotenoids (*e.g.*, lycopene and β-carotene).

2. Charged and Neutral Lipid Compositions

In certain embodiments, a lipid component of a composition may be uncharged or primarily uncharged. A lipid component of a composition may comprises one or more neutral lipids. A lipid component of a composition may also be substantially free of anionic and cationic lipids, such as certain phospholipids and cholesterol. In certain aspects, a lipid component of an uncharged or primarily uncharged lipid composition may comprise about 95%, about 96%, about 97%, about 98%, about 99% or 100% lipids without a charge, substantially uncharged lipid(s), and/or a lipid mixture with equal numbers of positive and negative charges.

In other aspects, a lipid composition may be charged. For example, charged phospholipids may be used for preparing a lipid composition according to the present invention and can carry a net positive charge or a net negative charge. In a non-limiting example, diacetyl phosphate can be employed to confer a negative charge on the lipid composition, and stearylamine can be used to confer a positive charge on the lipid composition.

3. Making Lipids

Lipids can be obtained from natural sources, commercial sources or chemically synthesized, as would be known to one of ordinary skill in the art. For example, phospholipids can be from natural sources, such as egg or soybean phosphatidylcholine, brain phosphatidic acid, brain or plant phosphatidylinositol, heart cardiolipin and plant or bacterial phosphatidylethanolamine. In another example, lipids suitable for use according to the present invention can be obtained from commercial sources. For example, the cationic lipid - 1,2-diacyl-sn-glycero-3-ethylphosphocholine and other lipids such as PEG lipids may be obtained from Avanti Polar Lipids, Inc., (Birmingham, AL.). In certain embodiments, stock solutions of lipids in chloroform or chloroform/methanol can be stored at about -20°C. Preferably, chloroform is used as the solvent since it is more readily evaporated than methanol.

D. Liposomes

In particular embodiments of the present invention, a lipid may comprise a liposome. A "liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes may be characterized as having vesicular structures with a bilayer membrane, generally comprising a phospholipid and an inner medium that generally comprises an aqueous composition.

A multilamellar liposome has multiple lipid layers separated by aqueous medium. They form spontaneously when lipids comprising phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Lipophilic molecules or molecules with lipophilic regions may also dissolve in or associate with the lipid bilayer.

In specific aspects, a lipid may be, for example, encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and a selected molecule or agent entrapped in a liposome, complexed with a liposome, *etc.*

The present invention provides a series of formulation for Effective Non-viral Gene an drug Delivery systems (ENGDs) comprising of phospholipids, lipids and polymers to form a liposome or microcapsule for the delivery of a molecule or an agent to a cell as a therapeutic modality.

1. Making Liposomes

In a preferred embodiment, the delivery compositions of the present invention are made using very simple steps. Example 1 describes the simplified procedure, wherein a cationic lipid - 1,2-diacyl-sn-glycero-3-ethylphosphocholine, a PEG-lated lipid and cationic polymers were mixed to produce the series of formulations of the present invention. It is contemplated that in the present invention, such reagents may be admixed using standard liquid mixing protocols and apparatus, as would be known to those of skill in the art.

Additionally, a liposome used according to the present invention can be made by different methods, as would be known to one of ordinary skill in the art. For example, a phospholipid (Avanti Polar Lipids, Alabaster, AL), such as for example the neutral phospholipid dioleoylphosphatidylcholine (DOPC), is dissolved in tert-butanol. The lipid(s) is then mixed with the other component(s). Tween 20 is added to the lipid mixture such that Tween 20 is about 5% of the composition's weight. Excess tert-butanol is added to this mixture such that the volume of tert-butanol is at least 95%. The mixture is vortexed, frozen in a dry ice/acetone bath and lyophilized overnight. The lyophilized preparation is stored at –20°C and can be used up to three months. When required the lyophilized liposomes are reconstituted in 0.9% saline.

Alternatively, a liposome can be prepared by mixing lipids in a solvent in a container, *e.g.*, a glass, pear-shaped flask. The container should have a volume ten-times greater than the volume of the expected suspension of liposomes. Using a rotary evaporator, the solvent is

removed at approximately 40°C under negative pressure. The solvent normally is removed within about 5 minutes to 2 hours, depending on the desired volume of the liposomes. The composition can be dried further in a desiccator under vacuum. The dried lipids generally are discarded after about 1 week because of a tendency to deteriorate with time.

Dried lipids can be hydrated at approximately 25-50 mM phospholipid in sterile, pyrogen-free water by shaking until all the lipid film is resuspended. The aqueous liposomes can be then separated into aliquots, each placed in a vial, lyophilized and sealed under vacuum.

In other alternative methods, liposomes can be prepared in accordance with other known laboratory procedures (*e.g.*, see Bangham *et al.*, 1965; Gregoriadis, 1979; Deamer and Uster, 1983; Szoka and Papahadjopoulos, 1978; each incorporated herein by reference in relevant part). These methods differ in their respective abilities to entrap aqueous material and their respective aqueous space-to-lipid ratios.

The dried lipids or lyophilized liposomes prepared as described above may be dehydrated and reconstituted in a solution of inhibitory peptide and diluted to an appropriate concentration with an suitable solvent, *e.g.*, DPBS. The mixture is then vigorously shaken in a vortex mixer. Unencapsulated additional materials, such as agents including but not limited to hormones, drugs, nucleic acid constructs and the like, are removed by centrifugation at 29,000 × g and the liposomal pellets washed. The washed liposomes are resuspended at an appropriate total phospholipid concentration, *e.g.*, about 50-200 mM. The amount of additional material or active agent encapsulated can be determined in accordance with standard methods. After determination of the amount of additional material or active agent encapsulated in the liposome preparation, the liposomes may be diluted to appropriate concentrations and stored at 4°C until use. A pharmaceutical composition comprising the liposomes will usually include a sterile, pharmaceutically acceptable carrier or diluent, such as water or saline solution.

The size of a liposome may vary depending on the method of synthesis. Liposomes as contemplated in the present invention can be a variety of sizes. In certain embodiments, the liposomes are small, *e.g.*, less than about 100 nm, about 90 nm, about 80 nm, about 70 nm, about 60 nm, or less than about 50 nm in external diameter. In preparing such liposomes, any protocol described herein, or as would be known to one of ordinary skill in the art may be used. Additional non-limiting examples of preparing liposomes are described in U.S. Patents 4,728,578, 4,728,575, 4,737,323, 4,533,254, 4,162,282, 4,310,505, and 4,921,706; International Applications PCT/US85/01161 and PCT/US89/05040; U.K. Patent Application GB 2193095 A; Mayer *et al.*, 1986; Mayhew *et al.*, 1987; Mayhew *et al.*, 1984; Cheng *et al.*, 1987; and Liposome Technology, Gregory Gregoriadis (Ed.), 1984, each incorporated herein by reference).

A liposome suspended in an aqueous solution is generally in the shape of a spherical vesicle, having one or more concentric layers of lipid bilayer molecules. Each layer consists of a parallel array of molecules represented by the formula XY, wherein X is a hydrophilic moiety and Y is a hydrophobic moiety. In aqueous suspension, the concentric layers are arranged such that the hydrophilic moieties tend to remain in contact with an aqueous phase and the hydrophobic regions tend to self-associate. For example, when aqueous phases are present both within and without the liposome, the lipid molecules may form a bilayer, known as a lamella, of the arrangement XY-YX. Aggregates of lipids may form when the hydrophilic and hydrophobic parts of more than one lipid molecule become associated with each other. The size and shape of these aggregates will depend upon many different variables, such as the nature of the solvent and the presence of other compounds in the solution.

The production of lipid formulations often is accomplished by sonication or serial extrusion of liposomal mixtures after (I) reverse phase evaporation (II) dehydration-rehydration (III) detergent dialysis and (IV) thin film hydration. In one aspect, a contemplated method for preparing liposomes in certain embodiments is heating sonicating, and sequential extrusion of the lipids through filters or membranes of decreasing pore size, thereby resulting in the formation of small, stable liposome structures. This preparation produces liposomal delivery compositions of appropriate and uniform size, which are structurally stable and produce maximal activity. Such techniques are well-known to those of skill in the art (see, for example Martin, 1990).

Once manufactured, lipid structures can be used to encapsulate compounds that are toxic (*e.g.*, chemotherapeutics) or labile (*e.g.*, nucleic acids) when in circulation. Liposomal encapsulation has resulted in a lower toxicity and a longer serum half-life for such compounds (Gabizon *et al.*, 1990).

Numerous disease treatments are using lipid based gene transfer strategies to enhance conventional or establish novel therapies, in particular therapies for treating hyperproliferative diseases. Advances in liposome formulations have improved the efficiency of gene transfer *in vivo* (Templeton *et al.*, 1997) and it is contemplated that liposomes are prepared by these methods. Alternate methods of preparing lipid-based formulations for nucleic acid delivery are described (W0 99/18933).

In another liposome formulation, an amphipathic vehicle called a solvent dilution microcarrier (SDMC) enables integration of particular molecules into the bi-layer of the lipid vehicle (U.S. Patent 5,879,703). The SDMCs can be used to deliver lipopolysaccharides, polypeptides, nucleic acids and the like. Of course, any other methods of liposome preparation

can be used by the skilled artisan to obtain a desired liposome formulation in the present invention.

2. Liposome Targeting

Association of a molecule or agent with a liposome may improve biodistribution and other properties of the molecule or agent. For example, liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Nicolau *et al.*, 1987). The feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells has also been demonstrated (Wong *et al.*, 1980). Successful liposome-mediated gene transfer in rats after intravenous injection has also been accomplished (Nicolau *et al.*, 1987).

It is contemplated that the delivery composition of the present invention may comprise additional materials for delivery to a tissue. For example, in certain embodiments of the invention, the lipid or liposome may be associated with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In another example, the lipid or liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the lipid may be complexed or employed in conjunction with both HVJ and HMG-1.

Targeted delivery is achieved by the addition of ligands without compromising the ability of these liposomes to deliver large amounts of a molecule or agent. It is contemplated that this will enable delivery to specific cells, tissues and organs. The targeting specificity of the ligand-based delivery systems are based on the distribution of the ligand receptors on different cell types. The targeting ligand may either be non-covalently or covalently associated with the lipid complex, and can be conjugated to the liposomes by a variety of methods.

The term "ligand" includes any molecule, compound or device with a reactive functional group and includes lipids, amphipathic lipids, carrier compounds, chelating moities, bioaffinity compounds, biomaterials, biopolymers, biomedical devices, analytically detectable compounds, therapeutically active compounds, enzymes, peptides, proteins, antibodies, immune stimulators, radiolabels, fluorogens, biotin, drugs, haptens, DNA, RNA, polysaccharides, liposomes, virosomes, micelles, immunoglobulins, functional groups, targeting agents, or toxins. The foregoing list is illustrative.

i. Biochemical Cross-linkers

Cross-linking reagents are used to form molecular bridges that tie together functional groups of two different molecules, *e.g.*, a stabilizing and a coagulating agent. To link two different proteins in a step-wise manner, hetero-bifunctional cross-linkers can be used that eliminate unwanted homopolymer formation.

Bifunctional cross-linking reagents have been extensively used for a variety of purposes including preparation of affinity matrices, modification and stabilization of diverse structures, identification of ligand and receptor binding sites, and structural studies. Homobifunctional reagents that carry two identical functional groups proved to be highly efficient in inducing cross-linking between identical and different macromolecules or subunits of a macromolecule, and linking of polypeptide ligands to their specific binding sites. Heterobifunctional reagents contain two different functional groups. By taking advantage of the differential reactivities of the two different functional groups, cross-linking can be controlled both selectively and sequentially. The bifunctional cross-linking reagents can be divided according to the specificity of their functional groups, *e.g.*, amino, sulfhydryl, guanidino, indole, carboxyl specific groups. Of these, reagents directed to free amino groups have become especially popular because of their commercial availability, ease of synthesis and the mild reaction conditions under which they can be applied. A majority of heterobifunctional cross-linking reagents contains a primary amine-reactive group and a thiol-reactive group.

Exemplary methods for cross-linking ligands to liposomes are described in U.S. Patent 5,603,872 and U.S. Patent 5,401,511, each specifically incorporated herein by reference in its entirety). Various ligands can be covalently bound to liposomal surfaces through the cross-linking of amine residues. Liposomes, in particular, multilamellar vesicles (MLV) or unilamellar vesicles such as microemulsified liposomes (MEL) and large unilamellar liposomes (LUVET), each containing phosphatidylethanolamine (PE), have been prepared by established procedures. The inclusion of PE in the liposome provides an active functional residue, a primary amine, on the liposomal surface for cross-linking purposes. Ligands such as epidermal growth factor (EGF) have been successfully linked with PE-liposomes. Ligands are bound covalently to discrete sites on the liposome surfaces. The number and surface density of these sites will be dictated by the liposome formulation and the liposome type. The liposomal surfaces may also have sites for non-covalent association. To form covalent conjugates of ligands and liposomes, cross-linking reagents have been studied for effectiveness and biocompatibility. Cross-linking reagents include glutaraldehyde (GAD), bifunctional oxirane (OXR), ethylene glycol diglycidyl ether (EGDE), and a water soluble carbodiimide, preferably 1-ethyl-3-(3-dimethylaminopropyl)

carbodiimide (EDC). Through the complex chemistry of cross-linking, linkage of the amine residues of the recognizing substance and liposomes is established.

In another example, heterobifunctional cross-linking reagents and methods of using the cross-linking reagents are described (U.S. Patent 5,889,155, specifically incorporated herein by reference in its entirety). The cross-linking reagents combine a nucleophilic hydrazide residue with an electrophilic maleimide residue, allowing coupling in one example, of aldehydes to free thiols. The cross-linking reagent can be modified to cross-link various functional groups and is thus useful for cross-linking polypeptides and sugars. Table 1 details certain hetero-bifunctional cross-linkers considered useful in the present invention.

TABLE 1
HETERO-BIFUNCTIONAL CROSS-LINKERS

Linker	Reactive Toward	Advantages and Applications	Spacer Arm Length\after cross-linking
SMPT	Primary amines Sulfhydryls	· Greater stability	11.2 A
SPDP	Primary amines Sulfhydryls	· Thiolation · Cleavable cross-linking	6.8 A
LC-SPDP	Primary amines Sulfhydryls	· Extended spacer arm	15.6 A
Sulfo-LC-SPDP	Primary amines Sulfhydryls	· Extended spacer arm · Water-soluble	15.6 A
SMCC	Primary amines Sulfhydryls	· Stable maleimide reactive group · Enzyme-antibody conjugation · Hapten-carrier protein conjugation	11.6 A
Sulfo-SMCC	Primary amines Sulfhydryls	· Stable maleimide reactive group · Water-soluble · Enzyme-antibody conjugation	11.6 A
MBS	Primary amines Sulfhydryls	· Enzyme-antibody conjugation · Hapten-carrier protein conjugation	9.9 A
Sulfo-MBS	Primary amines Sulfhydryls	· Water-soluble	9.9 A
SIAB	Primary amines Sulfhydryls	· Enzyme-antibody conjugation	10.6 A
Sulfo-SIAB	Primary amines Sulfhydryls	· Water-soluble	10.6 A

TABLE 1 HETERO-BIFUNCTIONAL CROSS-LINKERS			
SMPB	Primary amines Sulfhydryls	· Extended spacer arm · Enzyme-antibody conjugation	14.5 A
Sulfo-SMPB	Primary amines Sulfhydryls	· Extended spacer arm · Water-soluble	14.5 A
EDC/Sulfo-NHS	Primary amines Carboxyl groups	· Hapten-Carrier conjugation	0
ABH	Carbohydrates Nonselective	· Reacts with sugar groups	11.9 A

In instances where a particular polypeptide does not contain a residue amenable for a given cross-linking reagent in its native sequence, conservative genetic or synthetic amino acid changes in the primary sequence can be utilized.

ii. Targeting Ligands

The targeting ligand can be either anchored in the hydrophobic portion of the complex of the present invention or attached to reactive terminal groups of the hydrophilic portion of the complex. The targeting ligand can be attached to the composition of the present invention via a linkage to a reactive group, *e.g.*, on the distal end of the hydrophilic polymer. Preferred reactive groups include amino groups, carboxylic groups, hydrazide groups, and thiol groups. The coupling of the targeting ligand to the hydrophilic polymer can be performed by standard methods of organic chemistry that are known to those skilled in the art.

Targeting ligands are any ligand specific for a characteristic component of the targeted region. Preferred targeting ligands include proteins such as polyclonal or monoclonal antibodies, antibody fragments, or chimeric antibodies, enzymes, or hormones, or sugars such as mono-, oligo- and poly-saccharides (see, Heath *et al.,* 1986). Targeting ligands contemplated in the present invention may interact with integrins, proteoglycans, glycoproteins, receptors or transporters. Suitable ligands include any that are specific for cells of the target organ, or for structures of the target organ exposed to the circulation as a result of local pathology, such as tumors.

Targeting ligands are any ligand specific for a characteristic component of the targeted region. Such targeting ligands include proteins such as polyclonal or monoclonal antibodies, antibody fragments, or chimeric antibodies, enzymes, or hormones, or sugars such as mono-, oligo- and poly-saccharides (see, Heath *et al.,* 1986). For example, disialoganglioside GD2 is a tumor antigen that has been identified neuroectodermal origin tumors, such as neuroblastoma, melanoma, small-cell lung carcinoma, glioma and certain sarcomas (Mujoo *et al.,* 1996;

Schulz *et al.*, 1984). Liposomes containing anti-disialoganglioside GD2 monoclonal antibodies have been used to aid the targeting of the liposomes to cells expressing the tumor antigen (Montaldo *et al.*, 1999). In another non-limiting example, breast and gynecological cancer antigen specific antibodies are described in U.S. Patent 5,939,277, incorporated herein by reference. In a further non-limiting example, prostate cancer specific antibodies are disclosed in U.S. Patent 6,107,090, incorporated herein by reference. Thus, it is contemplated that the antibodies described herein or as would be known to one of ordinary skill in the art may be used to target specific tissues and cell types in combination with the compositions and methods of the present invention.

Still further, a molecule or agent may be delivered to a target cell via receptor-mediated delivery and/or targeting vehicles comprising a lipid or liposome. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis that will be occurring in a target cell. In view of the cell type-specific distribution of various receptors, this delivery method adds another degree of specificity to the present invention.

Thus, in certain aspects of the present invention, a ligand will be chosen to correspond to a receptor specifically expressed on the target cell population. A cell-specific molecule or agent for delivery and/or targeting to a cell may comprise a specific binding ligand in combination with a liposome. The molecule or agent to be delivered are housed within a liposome and the specific binding ligand is functionally incorporated into a liposome membrane. The liposome will thus specifically bind to the receptor(s) of a target cell and deliver the contents to a cell. Such systems have been shown to be functional using systems in which, for example, epidermal growth factor (EGF) is used in the receptor-mediated delivery of a nucleic acid to cells that exhibit upregulation of the EGF receptor.

In certain embodiments, a receptor-mediated delivery and/or targeting vehicles comprise a cell receptor-specific ligand and a binding agent. Others comprise a cell receptor-specific ligand to which the molecule or agent to be delivered has been operatively attached. For example, several ligands have been used for receptor-mediated gene transfer (Wu and Wu, 1987; Wagner *et al.*, 1990; Perales *et al.*, 1994; Myers, EPO 0273085), which establishes the operability of the technique. In another example, specific delivery in the context of another mammalian cell type has been described (Wu and Wu, 1993; incorporated herein by reference).

In still further embodiments, the specific binding ligand may comprise one or more lipids or glycoproteins that direct cell-specific binding. For example, lactosyl-ceramide, a galactose-terminal asialganglioside, have been incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes (Nicolau *et al.*, 1987). The asialoglycoprotein,

asialofetuin, which contains terminal galactosyl residues, also has been demonstrated to target liposomes to the liver (Spanjer and Scherphof, 1983; Hara *et al.*, 1996). The sugars mannosyi, fucosyl or N-acetyl glucosamine, when coupled to the backbone of a polypeptide, bind the high affinity manose receptor (U.S. Patent 5,432,260, specifically incorporated herein by reference in its entirety).

In another example, lactosyl ceramide, and peptides that target the LDL receptor related proteins, such as apolipoprotein E3 ("Apo E") have been useful in targeting liposomes to the liver (Spanjer and Scherphof, 1983; WO 98/0748).

Folate and the folate receptor have also been described as useful for cellular targeting (U.S Patent 5,871,727). In this example, the vitamin folate is coupled to the complex. The folate receptor has high affinity for its ligand and is overexpressed on the surface of several malignant cell lines, including lung, breast and brain tumors. Anti-folate such as methotrexate may also be used as targeting ligands. Transferrin mediated delivery systems target a wide range of replicating cells that express the transferrin receptor (Gilliland *et al.*, 1980).

E. Cancer Agents for Use with the Delivery System of the Invention

It is contemplated that the compositions of the present invention may be used to deliver a molecule or agent to a cell such as a cancer cell, for treatment of a cancer. This process involves contacting the cell(s) with the delivery composition comprising the molecule or agent.

The terms "contacted" and "exposed," when applied to a cell, tissue or organism, are used herein to describe the process by which the therapeutic compositions of the invention is delivered to a target cell, tissue or organism or are placed in direct juxtaposition with the target cell, tissue or organism. To achieve cell killing or stasis, an amount effective of the therapeutic composition is delivered to one or more cells to kill the cell(s) or prevent them from dividing. The therapeutic compositions of the present invention may be delivered to a subject in treatment cycles ranging from minutes to weeks to months.

Agents that may be delivered in the compositions of the present invention include but are not limited to chemotherapeutic agents, radiotherapeutic agents, immunotherapeutic agents, genetic therapy agents, hormonal agents, and other biological agents.

1. Anticancer Agents

In particular embodiments agents that may be combined with the delivery composition of the present invention for treatment of a hyperproliferative disease such as cancer, include anti-cancer agents. An anti-cancer agent is capable of negatively affecting cancer in a subject, for

example, by killing one or more cancer cells, inducing apoptosis in one or more cancer cells, reducing the growth rate of one or more cancer cells, reducing the incidence or number of metastases, reducing a tumor's size, inhibiting a tumor's growth, reducing the blood supply to a tumor or one or more cancer cells, promoting an immune response against one or more cancer cells or a tumor, preventing or inhibiting the progression of a cancer, or increasing the lifespan of a subject with a cancer. Anti-cancer agents include, for example, chemotherapy agents (chemotherapy), radiotherapy agents (radiotherapy), a surgical procedure (surgery), immune therapy agents (immunotherapy), genetic therapy agents (gene therapy), hormonal therapy, other biological agents (biotherapy) and/or alternative therapies.

a. Chemotherapeutic Agents

The term "chemotherapy" refers to the use of drugs to treat cancer. A "chemotherapeutic agent" is used to connote a compound or composition that is administered in the treatment of cancer. One subtype of chemotherapy known as biochemotherapy involves the combination of a chemotherapy with a biological therapy.

Chemotherapeutic agents include, but are not limited to, 5-fluorouracil, bleomycin, busulfan, camptothecin, carboplatin, chlorambucil, cisplatin (CDDP), cyclophosphamide, dactinomycin, daunorubicin, doxorubicin, estrogen receptor binding agents, etoposide (VP16), farnesyl-protein transferase inhibitors, gemcitabine, ifosfamide, mechlorethamine, melphalan, mitomycin, navelbine, nitrosurea, plicomycin, procarbazine, raloxifene, tamoxifen, taxol, temazolomide (an aqueous form of DTIC), transplatinum, vinblastine and methotrexate, vincristine, or any analog or derivative variant of the foregoing. These agents or drugs are categorized by their mode of activity within a cell, for example, whether and at what stage they affect the cell cycle. Alternatively, an agent may be characterized based on its ability to directly cross-link DNA, to intercalate into DNA, or to induce chromosomal and mitotic aberrations by affecting nucleic acid synthesis. Most chemotherapeutic agents fall into the following categories: alkylating agents, antimetabolites, anti-tumor antibiotics, corticosteroid hormones, mitotic inhibitors, and nitrosoureas, hormone agents, miscellaneous agents, and any analog or derivative variant thereof.

Chemotherapeutic agents and methods of administration, dosages, *etc.* are well known to those of skill in the art (see for example, the "Physicians Desk Reference," Goodman & Gilman's "The Pharmacological Basis of Therapeutics" and in "Remington's Pharmaceutical Sciences," incorporated herein by reference in relevant parts), and may be combined with the invention in light of the disclosures herein. Some variation in dosage will necessarily occur

depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Examples of specific chemotherapeutic agents and dose regimes are also described herein. Of course, all of these dosages and agents described herein are exemplary rather than limiting, and other doses or agents may be used by a skilled artisan for a specific patient or application. Any dosage in-between these points, or range derivable therein is also expected to be of use in the invention.

b. Alkylating agents

Alkylating agents are drugs that directly interact with genomic DNA to prevent the cancer cell from proliferating. This category of chemotherapeutic drugs represents agents that affect all phases of the cell cycle, that is, they are not phase-specific. Alkylating agents can be implemented to treat, for example, chronic leukemia, non-Hodgkin's lymphoma, Hodgkin's disease, multiple myeloma, and particular cancers of the breast, lung, and ovary. An alkylating agent, may include, but is not limited to, a nitrogen mustard, an ethylenimene, a methylmelamine, an alkyl sulfonate, a nitrosourea or a triazines.

They include but are not limited to: busulfan, chlorambucil, cisplatin, cyclophosphamide (cytoxan), dacarbazine, ifosfamide, mechlorethamine (mustargen), and melphalan. In specific aspects, the delivery composition of the present invention can be used to deliver any one or more of these alkylating agents to a cell to treat the cancer.

c. Antimetabolites

Antimetabolites disrupt DNA and RNA synthesis. Unlike alkylating agents, they specifically influence the cell cycle during S phase. They have used to combat chronic leukemias in addition to tumors of breast, ovary and the gastrointestinal tract. Antimetabolites can be differentiated into various categories, such as folic acid analogs, pyrimidine analogs and purine analogs and related inhibitory compounds. Antimetabolites include but are not limited to, 5-fluorouracil (5-FU), cytarabine (Ara-C), fludarabine, gemcitabine, and methotrexate.

d. Natural Products

Natural products generally refer to compounds originally isolated from a natural source, and identified has having a pharmacological activity. Such compounds, analogs and derivatives thereof may be, isolated from a natural source, chemically synthesized or recombinantly produced by any technique known to those of skill in the art. Natural products include such

categories as mitotic inhibitors, anti-tumor antibiotics, enzymes and biological response modifiers.

e. Anti-tumor Antibiotics

Anti-tumor antibiotics have both antimicrobial and cytotoxic activity. These drugs also interfere with DNA by chemically inhibiting enzymes and mitosis or altering cellular membranes. These agents are not phase specific so they work in all phases of the cell cycle. Thus, they are widely used for a variety of cancers. Examples of anti-tumor antibiotics include, but are not limited to, bleomycin, dactinomycin, daunorubicin, doxorubicin (adriamycin), plicamycin (mithramycin) and idarubicin. Widely used in clinical setting for the treatment of neoplasms these compounds generally are administered through intravenous bolus injections or orally.

f. Hormones and Antagonists

Hormonal therapy may also be used in conjunction with the present invention and/or in combination with any other cancer therapy or agent(s). The use of hormones may be employed in the treatment of certain cancers such as breast, prostate, ovarian, or cervical cancer to lower the level or block the effects of certain hormones such as testosterone or estrogen. This treatment is often used in combination with at least one other cancer therapy as a treatment option or to reduce the risk of metastases.

Progestins such as hydroxyprogesterone caproate, medroxyprogesterone acetate, and megestrol acetate have been used in cancers of the endometrium and breast. Estrogens such as diethylstilbestrol and ethinyl estradiol have been used in cancers such as breast and prostate. Antiestrogens such as tamoxifen have been used in cancers such as breast. Androgens such as testosterone propionate and fluoxymesterone have also been used in treating breast cancer. Antiandrogens such as flutamide have been used in the treatment of prostate cancer. Gonadotropin-releasing hormone analogs such as leuprolide have been used in treating prostate cancer. U.S. Patent 4,418,068, incorporated herein by reference, discloses antiestrogenic and antiandrogenic benzothiophenes, such as, for example, 6-hydroxy-2-(4-hydroxyphenyl)-3-[4-(2-piperidinoethoxy)benzoyl]benzo[b]thiophene, and esters, ethers, and salts thereof for the treatment of cancers such as prostate and breast cancer.

g. Miscellaneous Agents

Some chemotherapy agents do not qualify into the previous categories based on their activities. They include, but are not limited to, platinum coordination complexes, anthracenedione, substituted urea, methyl hydrazine derivative, adrenalcortical suppressant, amsacrine, L-asparaginase, and tretinoin. It is contemplated that they are included within the compositions and methods of the present invention for use in combination therapies.

An anthracenedione such as mitoxantrone has been used for treating acute granulocytic leukemia and breast cancer. A substituted urea such as hydroxyurea has been used in treating chronic granulocytic leukemia, polycythemia vera, essential thrombocytosis and malignant melanoma. A methyl hydrazine derivative such as procarbazine (N-methylhydrazine, M IH) has been used in the treatment of Hodgkin's disease. An adrenocortical suppressant such as mitotane has been used to treat adrenal cortex cancer, while aminoglutethimide has been used to treat Hodgkin's disease.

h. Radiotherapeutic Agents

Radiotherapeutic agents include radiation and waves that induce DNA damage for example, γ-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, radioisotopes, and the like. Therapy may be achieved by irradiating the localized tumor site with the above described forms of radiations. It is most likely that all of these agents effect a broad range of damage DNA, on the precursors of DNA, the replication and repair of DNA, and the assembly and maintenance of chromosomes.

Radiotherapeutic agents and methods of administration, dosages, *etc.* are well known to those of skill in the art, and may be combined with the invention in light of the disclosures herein. For example, dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 weeks), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

i. Surgery

Approximately 60% of persons with cancer will undergo surgery of some type, which includes, for example, preventative, diagnostic or staging, curative and palliative surgery. Surgery, and in particular a curative surgery, may be used in conjunction with other therapies, such as the present invention wherein the delivery composition delivers an agent or molecule to a cell or tissue.

Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised and/or destroyed. It is further contemplated that surgery may remove, excise or destroy superficial cancers, precancers, or incidental amounts of normal tissue. Treatment by surgery includes for example, tumor resection, laser surgery, cryosurgery, electrosurgery, and miscopically controlled surgery (Mohs' surgery). Tumor resection refers to physical removal of at least part of a tumor. Upon excision of part of all of cancerous cells, tissue, or tumor, a cavity may be formed in the body.

Further treatment of the tumor or area of surgery may be accomplished by perfusion, direct injection or local application of the area with an additional anti-cancer agent. Such treatment may be repeated, for example, about every 1, about every 2, about every 3, about every 4, about every 5, about every 6, or about every 7 days, or about every 1, about every 2, about every 3, about every 4, or about every 5 weeks or about every 1, about every 2, about every 3, about every 4, about every 5, about every 6, about every 7, about every 8, about every 9, about every 10, about every 11, or about every 12 months. These treatments may be of varying dosages as well.

j. Immunotherapeutic Agents

An immunotherapeutic agent generally relies on the use of immune effector cells and molecules to target and destroy cancer cells. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually effect cell killing. The antibody also may be conjugated to a drug or toxin (*e.g.*, a chemotherapeutic, a radionuclide, a ricin A chain, a cholera toxin, a pertussis toxin, *etc.*) and serve merely as a targeting agent. Such antibody conjugates are called immunotoxins, and are well known in the art (see U.S. Patents 5,686,072, 5,578,706, 4,792,447, 5,045,451, 4,664,911, and 5,767,072, each incorporated herein by reference). Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells.

In one aspect of immunotherapy, the tumor cell must bear some marker that is amenable to targeting, *i.e.*, is not present on the majority of other cells. Many tumor markers exist and any of these may be suitable for targeting delivery to a cell in the context of the present invention. Common tumor markers include carcinoembryonic antigen, prostate specific antigen, urinary tumor associated antigen, fetal antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, estrogen receptor, laminin receptor, *erb* B and p155.

i. Immune Stimulators

A specific aspect of immunotherapy is to use an immune stimulating molecule as an agent, or more preferably in conjunction with another agent, such as for example, a cytokines such as for example IL-2, IL-4, IL-12, GM-CSF, tumor necrosis factor; interferons alpha, beta, and gamma; F42K and other cytokine analogs; a chemokine such as for example MIP-1, MIP-1beta, MCP-1, RANTES, IL-8; or a growth factor such as for example FLT3 ligand.

Tumor necrosis factor (TNF; Cachectin) is a glycoprotein that kills some kinds of cancer cells, activates cytokine production, activates macrophages and endothelial cells, promotes the production of collagen and collagenases, is an inflammatory mediator and also a mediator of septic shock, and promotes catabolism, fever and sleep. Some infectious agents cause tumor regression through the stimulation of TNF production. TNF can be quite toxic when used alone in effective doses, so that the optimal regimens probably will use it in lower doses in combination with other drugs. Its immunosuppressive actions are potentiated by gamma-interferon, so that the combination potentially is dangerous. A hybrid of TNF and interferon-α also has been found to possess anti-cancer activity.

Another cytokine specifically contemplate is interferon alpha. Interferon alpha has been used in treatment of hairy cell leukemia, Kaposi's sarcoma, melanoma, carcinoid, renal cell cancer, ovary cancer, bladder cancer, non-Hodgkin's lymphomas, mycosis fungoides, multiple myeloma, and chronic granulocytic leukemia.

ii. Passive Immunotherapy

A number of different approaches for passive immunotherapy of cancer exist. They may be broadly categorized into the following: injection of antibodies alone; injection of antibodies coupled to toxins or chemotherapeutic agents; injection of antibodies coupled to radioactive isotopes; injection of anti-idiotype antibodies; and finally, purging of tumor cells in bone marrow.

Preferably, human monoclonal antibodies are employed in passive immunotherapy, as they produce few or no side effects in the patient. However, their application is somewhat limited by their scarcity and have so far only been administered intralesionally. For example, human monoclonal antibodies to ganglioside antigens have been administered intralesionally to patients suffering from cutaneous recurrent melanoma (Irie and Morton, 1986). Regression was observed in six out of ten patients, following, daily or weekly, intralesional injections. In

another study, moderate success was achieved from intralesional injections of two human monoclonal antibodies (Irie *et al.*, 1989).

It may be favorable to administer more than one monoclonal antibody directed against two different antigens or even antibodies with multiple antigen specificity. Treatment protocols also may include administration of lymphokines or other immune enhancers (Bajorin *et al.*, 1988).

iii. Active Immunotherapy

In active immunotherapy, an antigenic peptide, polypeptide or protein, or an autologous or allogenic tumor cell composition or "vaccine" is administered, generally with a distinct bacterial adjuvant (Ravindranath and Morton, 1991; Morton *et al.*, 1992; Mitchell *et al.*, 1990; Mitchell *et al.*, 1993). In melanoma immunotherapy, those patients who elicit high IgM response often survive better than those who elicit no or low IgM antibodies (Morton *et al.*, 1992). IgM antibodies are often transient antibodies and the exception to the rule appears to be anti-ganglioside or anticarbohydrate antibodies.

k. Genetic Therapy Agents

A tumor cell resistance to agents, such as chemotherapeutic and radiotherapeutic agents, represents a major problem in clinical oncology. One goal of current cancer research is to find ways to improve the efficacy of one or more anti-cancer agents by combining such an agent with gene therapy. In the context of the present invention, it is contemplated that a non-viral delivery system would be used to deliver a gene to a cell or tissue for treatment of a cancer.

i. Inducers of Cellular Proliferation

In one embodiment of the present invention, it is contemplated that anti-sense mRNA directed to a particular inducer of cellular proliferation may be used to prevent expression of the inducer of cellular proliferation. The proteins that induce cellular proliferation further fall into various categories dependent on function. The commonality of all of these proteins is their ability to regulate cellular proliferation. The delivery composition of the present invention may be used to target these proteins to the cancer cell as a treatment therapy.

For example, a form of PDGF, the sis oncogene, is a secreted growth factor. Oncogenes rarely arise from genes encoding growth factors, and at the present, sis is the only known naturally-occurring oncogenic growth factor.

The proteins FMS, ErbA, ErbB and neu are growth factor receptors. Mutations to these receptors result in loss of regulatable function. For example, a point mutation affecting the transmembrane domain of the Neu receptor protein results in the neu oncogene. The erbA oncogene is derived from the intracellular receptor for thyroid hormone. The modified oncogenic ErbA receptor is believed to compete with the endogenous thyroid hormone receptor, causing uncontrolled growth.

The largest class of oncogenes includes the signal transducing proteins (*e.g.*, Src, Abl and Ras). The protein Src is a cytoplasmic protein-tyrosine kinase, and its transformation from proto-oncogene to oncogene in some cases, results via mutations at tyrosine residue 527. In contrast, transformation of GTPase protein ras from proto-oncogene to oncogene, in one example, results from a valine to glycine mutation at amino acid 12 in the sequence, reducing ras GTPase activity.

Other proteins such as Jun, Fos and Myc are proteins that directly exert their effects on nuclear functions as transcription factors.

ii. Inhibitors of Cellular Proliferation

In certain embodiments of the present invention, the delivery of a molecule involved in the restoration of the activity of an inhibitor of cellular proliferation is contemplated. Tumor suppressor oncogenes function to inhibit excessive cellular proliferation. The inactivation of these genes destroys their inhibitory activity, resulting in unregulated proliferation. The tumor suppressors p53, p16 and C-CAM are described below.

High levels of mutant p53 have been found in many cells transformed by chemical carcinogenesis, ultraviolet radiation, and several viruses. The p53 gene is a frequent target of mutational inactivation in a wide variety of human tumors and is already documented to be the most frequently mutated gene in common human cancers. It is mutated in over 50% of human NSCLC (Hollstein *et al.*, 1991) and in a wide spectrum of other tumors.

The p53 gene encodes a 393-amino acid phosphoprotein that can form complexes with host proteins such as large-T antigen and E1B. The protein is found in normal tissues and cells, but at concentrations which are minute by comparison with transformed cells or tumor tissue

Wild-type p53 is recognized as an important growth regulator in many cell types. Missense mutations are common for the p53 gene and are essential for the transforming ability of the oncogene. A single genetic change prompted by point mutations can create carcinogenic p53. Unlike other oncogenes, however, p53 point mutations are known to occur in at least 30 distinct codons, often creating dominant alleles that produce shifts in cell phenotype without a

reduction to homozygosity. Additionally, many of these dominant negative alleles appear to be tolerated in the organism and passed on in the germ line. Various mutant alleles appear to range from minimally dysfunctional to strongly penetrant, dominant negative alleles (Weinberg, 1991).

Another inhibitor of cellular proliferation is p16. The major transitions of the eukaryotic cell cycle are triggered by cyclin-dependent kinases, or CDK's. One CDK, cyclin-dependent kinase 4 (CDK4), regulates progression through the G_1. The activity of this enzyme may be to phosphorylate Rb at late G_1. The activity of CDK4 is controlled by an activating subunit, D-type cyclin, and by an inhibitory subunit, the $p16^{INK4}$ has been biochemically characterized as a protein that specifically binds to and inhibits CDK4, and thus may regulate Rb phosphorylation (Serrano *et al.*, 1993; Serrano *et al.*, 1995). Since the $p16^{INK4}$ protein is a CDK4 inhibitor (Serrano, 1993), deletion of this gene may increase the activity of CDK4, resulting in hyperphosphorylation of the Rb protein. p16 also is known to regulate the function of CDK6.

$p16^{INK4}$ belongs to a newly described class of CDK-inhibitory proteins that also includes $p16^{B}$, p19, $p21^{WAF1}$, and p27 ^{KIP1}. The $p16^{INK4}$ gene maps to 9p21, a chromosome region frequently deleted in many tumor types. Homozygous deletions and mutations of the $p16^{INK4}$ gene are frequent in human tumor cell lines. This evidence suggests that the $p16^{INK4}$ gene is a tumor suppressor gene. This interpretation has been challenged, however, by the observation that the frequency of the $p16^{INK4}$ gene alterations is much lower in primary uncultured tumors than in cultured cell lines (Caldas *et al.*,1994; Cheng *et al.*,1994; Hussussian *et al.*, 1994; Kamb *et al.*, 1994; Mori *et al.*, 1994; Okamoto *et al.*, 1994; Nobori *et al.*, 1995; Orlow *et al.*, 1994; Arap *et al.*, 1995). Restoration of wild-type $p16^{INK4}$ function by transfection with a plasmid expression vector reduced colony formation by some human cancer cell lines (Okamoto, 1994; Arap, 1995).

Other genes that may be employed according to the present invention include Rb, APC, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, zac1, p73, VHL, MMAC1 / PTEN, DBCCR-1, FCC, rsk-3, p27, p27/p16 fusions, p21/p27 fusions, anti-thrombotic genes (*e.g.*, COX-1, TFPI), PGS, Dp, E2F, *ras, myc, neu, raf, erb, fms, trk, ret, gsp, hst, abl*, E1A, p300, genes involved in angiogenesis (*e.g.*, VEGF, FGF, thrombospondin, BAI-1, GDAIF, or their receptors) and MCC.

iii. Regulators of Programmed Cell Death

In certain embodiments, it is contemplated that agents that stimulate apoptosis may be used to promote the death of diseased or undesired tissue. Such agents may be delivered using a composition of the present invention. Apoptosis, or programmed cell death, is an essential

process for normal embryonic development, maintaining homeostasis in adult tissues, and suppressing carcinogenesis (Kerr *et al.*, 1972). The Bcl-2 family of proteins and ICE-like proteases have been demonstrated to be important regulators and effectors of apoptosis in other systems. The Bcl-2 protein, discovered in association with follicular lymphoma, plays a prominent role in controlling apoptosis and enhancing cell survival in response to diverse apoptotic stimuli (Bakhshi *et al.*, 1985; Cleary and Sklar, 1985; Cleary *et al.*, 1986; Tsujimoto *et al.*, 1985; Tsujimoto and Croce, 1986). The evolutionarily conserved Bcl-2 protein now is recognized to be a member of a family of related proteins, which can be categorized as death agonists or death antagonists.

Subsequent to its discovery, it was shown that Bcl-2 acts to suppress cell death triggered by a variety of stimuli. Also, it now is apparent that there is a family of Bcl-2 cell death regulatory proteins which share in common structural and sequence homologies. These different family members have been shown to either possess similar functions to Bcl-2 (*e.g.*, Bcl_{XL}, Bcl_W, Bcl_S, Mcl-1, A1, Bfl-1) or counteract Bcl-2 function and promote cell death (*e.g.*, Bax, Bak, Bik, Bim, Bid, Bad, Harakiri).

F. Pharmaceutical Preparations

Pharmaceutical aqueous compositions of the present invention comprise the delivery composition and an effective amount of one or more of a selected molecule(s) or agent(s) dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. The phrases "pharmaceutically or pharmacologically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a human. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The actual dosage amount of a selected molecule(s) or agent(s) with the delivery composition of the present invention to be administered to a patient can be determined by physical and physiological factors such as body weight, severity of condition, idiopathy of the patient and on the route of administration. With these considerations in mind, the dosage of a lipid composition for a particular subject and/or course of treatment can readily be determined.

The present invention can be administered intravenously, intradermally, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostaticaly, intrapleurally, intratracheally, intranasally, intravitreally, intravaginally, rectally, topically, intratumorally, intramuscularly, intraperitoneally, subcutaneously, intravesicularlly, mucosally, intrapericardially, orally, topically, locally and/or using aerosol, injection, infusion, continuous infusion, localized perfusion bathing target cells directly or via a catheter and/or lavage. For example, the delivery composition and selected molecule or agent may be formulated for parenteral administration, *e.g.,* formulated for injection via the intravenous, intramuscular or subcutaneous routes, though other routes such aerosol administration may be used. The preparation of an aqueous composition that contains the delivery composition and at least one selected molecule or agent as an active ingredient will be known to those of skill in the art in light of the present disclosure, as exemplified by Remington's Pharmaceutical Sciences, 16th Ed. Mack Publishing Company, 1980, incorporated herein by reference. Moreover, for human administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for preparing solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified. The compositions will be sterile, be fluid to the extent that easy syringability exists, stable under the conditions of manufacture and storage, and preserved against the contaminating action of microorganisms, such as bacteria and fungi. It will be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less that 0.5 ng/mg protein.

Although it is most preferred that solutions of the delivery composition and selected molecule or agent be prepared in sterile water containing other non-active ingredients, made suitable for injection, solutions of such active ingredients can also be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose, if desired. Dispersions can also be prepared in liquid polyethylene glycols, and mixtures thereof and in oils. The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid,

thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

It is particularly contemplated that suitable pharmaceutical compositions will generally comprise, but are not limited to, from about 10 to about 100 mg of the desired molecule or agent admixed with an acceptable pharmaceutical diluent or excipient, such as a sterile aqueous solution, to give a final concentration of about 0.25 mg/ml to about 2.5 mg/ml with respect to the conjugate, in, for example, 0.15 M NaCl aqueous solution at pH 7.5 to 9.0. The preparations may be stored frozen at –10°C to -70°C for at least 1 year.

G. KITS

Any of the compositions described herein may be comprised in a kit. In a non-limiting example, the delivery composition and a selected molecule or agent, may be comprised in a kit. The kits will thus comprise, in suitable container means, the delivery composition of the present invention, and/or a selected molecule or agent. Preferably, the kits may comprise of one or more of the following: cationic lipids: 1,2-diacyl-sn-glycero-3-ethylphosphocholine; a lipid and a cationic polymer. The preferred lipid is 1,2-diacyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] or 1,2-diacyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] but is not limited to such. The preferred cationic polymer is at least any two of the following list of cationic polymers: polyethyleneimine, polylysine or protamine sulfate. A preferred agent would be one or more therapeutic or targeting agents of a particular disease.

The kits may comprise a suitably aliquot of the delivery composition of the present invention and a selected molecule or agent. The components of the kits may be packaged either in aqueous media or in lyophilized form. The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which a component may be placed, and preferably, suitably aliquoted. Where there are more than one component in the kit, the kit also will generally contain a second, third or other additional container into which the additional components may be separately placed. However, various combinations of components may be comprised in a vial. The kits of the present invention also will typically include a means for containing the various components, or pre-made delivery compositions and/or selected molecule(s) or agent(s), and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

The kit may contain the compositions of the present invention, preferably in a dehydrated form, with instructions for their rehydration and administration. In some embodiments, the components comprising the delivery composition and/or the molecule(s) or agent(s) of interest, may have a targeting moiety attached to their respective surfaces. Methods of attaching targeting moieties (for example, antibodies, proteins) to various components such as, for example, a lipid are known to those of skill in the art.

H. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Formulations

ENGD (Effective Non-viral Gene and drug Delivery system) is a series of formulations. These formulations are composed of the same amounts of cationic phospholipids, polymer linked lipids and other lipids, but different cationic polymers and different combination of cationic

polymers. The dosage form mainly belongs to liposome or microcapsule. The specific components of the formulation of the present invention are: cationic lipids: 1,2-diacyl-sn-glycero-3-ethylphosphocholine (Avanti Polar Lipids Inc.); PEG lipids:1,2-diacyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] or 1,2-diacyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (Avanti Polar Lipids Inc.); and cationic polymers: polyethyleneimine (MW 2,000~ 25,000); polylysine (MW 10,000 ~ 110,000); protamine sulfate (MW 3,000~5,000). Manufacturer Sigma Aldrich, MO.

The optimal compositions that may be used in the present invention are:

Polymer 1:Polymer 2 (mol):

Polylysine:Protamine = 0.3: 1 (0.1~1: 1)

PEI:Protamine = 0.02 : 1 (0.01~0.2: 1)

PEI:Protamine:Polylysine = 0.01: 0.3: 1 (0.002~0.02: 0.05~0.5: 1)

Cationic polymers:DNA (w/w) = 2~10: 0.1~5

PEG lipid:DNA (w/w) = 0.2~5: 0.1~5

Cationic lipid:DNA = 0.2~10: 0.1~5

The composition of the present invention may be prepared in the following manner: polymers and DNA are added to Tris-HCL or phosphate buffer solution at pH 6, and the solution is stirred gently for 30 seconds; three times each. The PEG-lipid, cationic lipid and (other lipids) are then dissolved in an organic solvent, methanol:chloroform 1:9 (v/v), on a rotating evaporator to make a thin-lipid film. The lipid film is then hydrated with the polymer-DNA particle solution. The liposomal formulation is next passed through a 0.22 μm membrane filter. The formulation of the liposomal DNA is purified by passing the products through a Qiagen resin column and Sephadex column.

EXAMPLE 2

Transfection Efficiency of ENGD-GFP

To determine the delivery capability of ENGD *in vitro*, ENGD was compared with commercial transfection reagents such as lipofectamine and lipofectine in various human cancer cell lines. Cells were cultured for 24 h in 6-well plates with 1 ml/well of medium containing 10% fetal bovine serum (FBS) (Life Technologies; Gaithersburg, MD) until 60-70% confluence was reached. Green fluorescence protein expression plasmid (GFP) was used as reporter gene. The carrier-GFP (ENGD-GFP, lipofectamine-GFP or lipofectine-GFP complex) with optimal

carrier to DNA ratio was directly added into the culture plates at a ratio of 2 μg DNA/10^6 cells. Forty-eight hours later, the transfection efficiency was determined by counting the GFP-positive cells under a fluorescence microscope and expressing the result as a percentage of total cells. Six random fields with >200 cells/field were counted for each sample. The results shown in FIG. 1 are mean standard deviation of three independent experiments. The overall results of this experiment demonstrated that ENGDs are much more efficient than the commercial transfection reagents in transfecting cells in serum containing cell cultures. Additionally, of the ENGDs tested, ENGD1 which is similar to ENGD5 proved to be better than the rest of the formulations.

EXAMPLE 3

Sustained Release Function of ENGD

The ability of ENGD to sustained release function compared with non-polymer liposomes was determined. A fluorescent compound Calcien obtained from Sigma, MO., (chemical reagents probe) was encapsulated into ENGDs and a control cationic liposome DP3 comprising the formulation of 1,2-dipalmitoyl-sn-glycero-3-ethylphosphocholine (DPEP) and dioleoyl 1,2-diacyl-sn-glycero-3-phosphoethanolamine (DOPE) at a weight ratio of 3:1 (particle size 60-110 nm). The Calcien containing formulations were purified with Sephadex G200 columns. The total Calcien concentrations of each formulation was determined by a fluorescence spectrophotometer. All formulations were diluted 1000x with medium containing 10% FBS at room temperature. At designed timepoints 3 mls of sample from each diluted formulation was taken and the released calcein was quantitatively determined with the fluorescence spectrophotometer. The results shown in FIG. 2 are mean standard deviation of three independent experiments. The overall results of this experiment demonstrated that ENGD has significant sustain release function compared to conventional liposomes.

EXAMPLE 4

Size of ENGD-DNA Complex Particles

The size of the ENGD-DNA complex particles in formulations of the present invention was determined. The ENGD-DNA size is stable for more than 2 days at room temperature. ENGD-GFP was suspended into PBS (pH 7.4) at room temperature. The DNA concentration is 40 μg/ml. At different timepoints, the sizes of the complex particles in each formulation were

measured with a dynamic light scattering (DLS) particle sizer. The results shown in FIG. 3 are the mean ± the standard deviation.

EXAMPLE 5

ENGD Efficiently Delivers a Reporter Gene

The ability of ENGD to efficiently deliver a reporter gene into a solid tumor and other organs in mice through intravenous injection was examined. The S.C. KB (head and neck cancer) and S.C. H358 (lung cancer) tumor models in nude mice were established. When the tumors were 0.6-0.8 cm in diameter, ENGD-Luc (60 μg DNA/mouse) was injected into the tail vein. Thirty-six hours later, the mice were killed, the proteins in the tumors and various organs were extracted, and the luciferase intensity in 100 mg of tissue was determined with a luminometer. Lipofectamine-Luc was used as a control in H358 tumor model, but no detectable luciferase in lipofectamine injected mice was found. The data shown in FIG. 4 are the mean ± the standard deviation from 5 mice. The overall results of this experiment demonstrated that ENGDs are able to deliver a gene to solid tumors and most organs through intravenously injection, but lipofectamine cannot.

EXAMPLE 6

ENGD-p53 Significantly Inhibits Growth of Human Solid Tumors

The ability of systemically injected ENGD-p53 to significantly inhibit growth of human solid tumors implanted in nude mice was examined. Nude mice were separately inoculated with 1-2 x 10^6 of different human cancer cells (KB, head and neck, H358, lung, and MCF-7 breast) subcutaneously. Two to three weeks later, when most tumors exceeded 5 mm in diameter, the tumor-bearing mice were randomly divided into 3-4 groups with 5 mice in each group. The mice in all treatment groups received intravenous injections of Lipofectamine-p53, ENGD1-p53, or ENGD5-p53 every 3 days for 5 times, each dose was 35 μg DNA/mouse The mice in the control groups were injected with the same dose of the ENGD1-vector. The tumor volume was measured weekly. The results are shown in FIGS 5A-5C. All statistical tests used in this study are two-sided log rank statistical tests. The overall results of this experiment demonstrated that ENGD-p53 is able to inhibit growth of different solid tumors after intravenous injection, but lipofectamine failed to do so ($P < 0.01$).

EXAMPLE 7

ENGD Efficiently Delivers Genes and Chemical Compounds *In Vivo*

It was investigated whether ENGD can efficiently deliver a reporter gene or chemical compound of low molecular weight to metastasis SCLC tumors in human SCLC xenografts. Early metastasis is one of the characteristics of human small cell lung carcinoma (SCLC). Systemic therapy is generally required for treating patients having SCLC.

The metastatic SCLC tumor model was made by implanting the human SCLC cell line NCI-H446 intravenously into nude mice (3×10^6 cells/mouse). Two to three weeks after implantation metastatic tumor nodules were observed in the lung, liver, diaphragm, and mesentery of mice.

To determine whether ENGD can efficiently deliver a gene to metastatic SCLC tumors ENGD was used to carry a luciferase reporter gene (luc) driven by a CMV promoter (FIG. 6). Nude mice (female, 6-7 weeks old, from Harland) were intravenously implanted with the human small cell lung carcinoma cell line NCI-H446 (3×10^6 cells/mouse). Three weeks later, five mice received 3-daily i.v. injections of the CMV-promoter driven luciferase gene entrapped in ENGD (ENGD-*luc*) (FIG. 6). The dose was 30 μg DNA/mouse/injection. Forty hours after the final injection, the mice were anesthetized and exsanguinated. The largest tumor nodules (≥2 mm in diameter) which were found in the liver and lung were isolated as metastatic tumors. Other organs and the liver tissue without visible tumor were taken as normal tissues. The tissues were homogenized with luciferase lysis buffer and the suspension centrifuged. Luciferase activity in the supernatant was determined using a luminometer. The luciferase activity in 100 mg of tissue was used as a criterion to measure gene expression in different tissues. The results as shown in FIG. 6 indicate that in an *in vivo* model ENDG can effectively deliver a gene to a metastatic tumor.

To determine the capability of ENGD for delivering small molecules to metastatic tumors 5 mice were given a single i.v. injection of ENGD-Calcein (4 μmol/mouse; FIG. 7). Sixteen hours later, the normal organs and metastatic tumors were obtained using the same procedure as described above. The tissues were homogenized with a phosphate buffer (pH 6.8). The fluorescent intensity in the supernatants was determined using a fluorescence spectrophotometer after centrifugation. The results as shown in FIG. 7 indicate that in an *in vivo* model ENDG can effectively deliver a small chemical compound to a metastatic tumor. The quantitative results obtained are based on a standard curve of Intensity verses μmol of Calcein.

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All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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